

The *ponA* Gene of *Enterococcus faecalis* JH2-2 Codes for a Low-Affinity Class A Penicillin-Binding Protein

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A soluble derivative of the *Enterococcus faecalis* JH2-2 class A PBP1 (*PBP1) was overproduced and purified. It exhibited a glycosyltransferase activity on the *Escherichia coli* ¹⁴C-labeled lipid II precursor. As a DD-peptidase, it could hydrolyze thiolester substrates with efficiencies similar to those of other class A penicillin-binding proteins (PBPs) and bind β -lactams, but with k_2/K (a parameter accounting for the acylation step efficiency) values characteristic of penicillin-resistant PBPs.

Bacteria have from 2 to 16 penicillin-binding proteins (PBPs) that fulfill different physiological functions and that are classified as low-molecular-mass (LMM) or high-molecular-mass (HMM) proteins (ca. 40 kDa and between 50 and 100 kDa, respectively). The LMM proteins are supposed to modify the degree of peptidoglycan (PG) cross-linking. The multimodular HMM PBPs are essential proteins subdivided into two classes. Class A proteins are bimodular, bifunctional enzymes. Their N-terminal module catalyzes transglycosylation reactions leading to the elongation of the glycan chains, and their penicillin-binding C-terminal module conducts transpeptidation reactions, ensuring the closure of the peptide bridges. The N-terminal module of class B proteins has no known enzymatic activity. It seems, however, to be needed for morphogenesis. As for class A proteins, their penicillin-binding C-terminal module catalyzes transpeptidation reactions. Primary structures of class A and class B N-terminal modules are easily distinguished by their different conserved amino acid motifs (five and three, respectively) (16, 17).

The number of class A PBPs may vary from species to species. For example, *Staphylococcus aureus* has only one (PBP2) (16), *Streptococcus pneumoniae* and *Escherichia coli* have three (PBP1a, -1b, and -2a and PBP1a, -1b, and -1c, respectively) (31, 41), and *Bacillus subtilis* has at least four (PBP1, -2c, -2d, and -4) (35). When several coexist in the same cell, each protein seems individually dispensable. However, in *E. coli* and *S. pneumoniae*, the absence of a specific pair of class A PBPs is not tolerated (20, 24, 28, 31, 39). In contrast, inactivation of all class A PBPs in *B. subtilis* and *Enterococcus faecalis* does not affect their viability but reduces their growth rates (4, 35). Thus, it is still premature to attribute a specific role to each of the class A PBPs.

From a biochemical point of view, the glycan chain polymerization reaction is now better understood. Most information was obtained by measuring glycan polymerization in intact cells

with radiolabeled hexosamine or amino acid residues or in ether- or toluene-permeabilized cells or crude cell wall or membrane preparations with peptidoglycan nucleotides or lipid II precursors (for a review, see reference 41). Purified soluble class A PBPs of *S. pneumoniae* (PBP1a, -1b, and -2a) and *E. coli* (PBP1b) were only recently obtained. Native and mutated derivatives were used to establish kinetic parameters of the glycosyltransferase reaction in the presence of nondansylated or dansylated lipid II precursors and to determine the importance of particular amino acid residues in that catalysis. Binding and/or inhibitory capacity of moenomycin and vancomycin antibiotics was determined. Acylation kinetics of the transpeptidase module of some of these PBPs with β -lactams were also analyzed (8, 9, 10, 11, 40, 43).

Enterococci are gram-positive bacteria that have recently emerged as nosocomial pathogens (6). Their opportunistic behavior is greatly enhanced by their intrinsic resistance to β -lactams and aminoglycosides and by their great ability to acquire genetic determinants that confer resistance to all classes of antibiotics: tetracyclines, aminoglycosides (high resistance levels), glycopeptides, and chloramphenicol (21). A better understanding of the intrinsic resistance mechanisms as well as of the PG synthetic machinery and more specifically of the class A PBPs is urgently needed to find new potential antibiotic targets that could reduce their spread.

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Isolation of the *ponA* gene of *E. faecalis* JH2-2. *E. faecalis* JH2-2 (Fus^r Rif^r strain derived from the nonhemolytic clinical JH2 strain) (22) grown without aeration in brain heart infusion broth up to the end of the exponential phase was used to isolate the genomic DNA as reported previously (27). Nucleotide sequences encoding class A PBPs of *S. pneumoniae*, *Streptococcus oralis*, *Streptococcus pyogenes*, and *S. aureus* were included in a multiple alignment used to synthesize two degenerate oligonucleotides, 5'-GAAGA(C,T)(A,C)A(T,A)CG(T,C)TTCT(T,A)(C,T)(G,A)A3' and 5'-A(A,T)(G,A)CTTC(C,T)TGAGC(C,T)TTACG3', that encoded or were complementary to the sequences encoding class A conserved motif 1, ED(H, K, N)

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TABLE 1. Oligonucleotides designed for cloning, sequencing, or expressing the *ponA* gene of *E. faecalis* JH2-2

Name	Sequence ^c (5'–3')	Position in AJ302065 sequence
EF _{V583} Motif5 ^a	GTCGTACATTGTGTAAAGTACAACATCCCGGCGTTC	2505–2470
EF _{V583} UP1	GAATGCGACAGTTTCATCGAAGCTTTACG ^b	1932–1960
EF _{V583} Motif3 ^a	ATGCTTCTTGTGCTTTCCGTTTCAAGGTTTGGT	2245–2213
EF _{V583} Motif3R	GACCAAACCTTGAAACGGAAGCACAAGAAGCATGG	2212–2247
EF-RP2 ^a	CAATTCTTTGTGGCTTGTCAATTCCACCCAG ^b	4244–4214
EF-RP3 ^a	GGTAAACTTGTACATTTGGTACCTCATAGGC ^b	3776–3745
EF-RP4 ^a	GTTAGAAGTCCCTGTTTTGGCCGCTTGG ^b	3477–3449
EF-RP5 ^a	TTTATCCACCATGAGGCGACCTGTC ^b	2979–2955
EF-Tpase1	CAATCATGGATTATGGTCCCGCAATTGAGA ^b	2912–2941
EF- <i>ponA</i> ^a	CGATGAGGTGTGGATGCATGTCTAGCAGC ^b	1767–1738
pET- <i>EcoRI</i>	TGGTTCGAATTCGACAAGCCCCAAAATTAGAAGATGAC	1900–1926
pET- <i>NotI</i>	GGTGAGCGGCCGCTTATTATGCTGCTTTATTTTC	4047–4029

^a Oligonucleotide on the complementary strand.^b Cy5 primer.^c Underlined sequences are *EcoRI* and *NotI* restriction sites.

RF(F, Y)(D, N, E), and motif 3, RKAQE(A, V)(W, Y), respectively (16, 17). A PCR performed with these primers on the genomic DNA of *E. faecalis* JH2-2 amplified a 183-bp fragment, the translated sequence of which contained the conserved GGSTLTQQ motif 2 found in class A PBPs. This 183-bp sequence was used as a probe for homology searches (BLAST program) in the genome of *E. faecalis* V583, the sequence of which became available at that time in The Institute for Genome Research (TIGR) database (<http://www.tigr.org>). A 2,183-bp sequence (1,000 bp on each side of the probe) was identified in contig 6429. The open reading frame (ORF) was named *ponA* by reference to the nomenclature used for the corresponding gene in *E. coli* (5). The 183-bp fragment conjugated with alkaline phosphatase (according to the instructions of the ALKPHOS system user's guide; Amersham Biosciences, Roosendaal, The Netherlands) was then prepared and tested by hybridization assays on different restriction digests of the JH2-2 genomic DNA transferred on Hybond N+ membranes (Amersham Biosciences). Single positive signals were detected in the PstI, the HindIII, and the BamHI digests at the levels of 4.5-, 4-, and 3.5-kb fragments, respectively.

PstI 4- to 5.5-kb fragments were isolated by electrophoresis, excised from the gel, purified with the help of the GeneClean spin kit (Bio 101 Systems, PolyLab, Antwerp, Belgium), and cloned into pUC18 (Amersham Biosciences). Recombinant plasmids extracted with the GFX MicroPlasmid Prep kit (Amersham Biosciences) from ~300 colonies of *E. coli* DH5 α (Amersham Biosciences) grown in Luria-Bertani medium containing ampicillin (100 μ g/ml) were screened by PCR with the EF_{V583}Motif5, EF_{V583}UP1, EF_{V583}Motif3, and EF_{V583}Motif3R primers (Table 1), the sequences of which derived from contig 6429. One clone yielded PCR fragments of the expected sizes with three primer combinations. The 4.5-kb insert found in the recombinant plasmid pDML1636 was completely sequenced on both strands with the Labstation Thermo Sequenase sequencing kit (Amersham Biosciences) with the Cy5-labeled M13 universal or reverse primers and Cy5-labeled oligonucleotides listed in Table 1. Electrophoresis was performed on an ALF Express DNA sequencer (Amersham Biosciences). The nucleotide sequences were introduced in

GELASSEMBLE (32), and homology searches in the SWISS-PROT, PIR, GENPEPT, and TIGR databases were made with FASTA or BLAST (3).

The *E. faecalis ponA* gene extends from nucleotide (nt) 1711 to nt 4044. It is preceded by a putative promoter located in the upstream ORF, 152 nt away from the initial ATG and followed by a sequence presenting a dyad symmetry (nt 4089 to 4128) that could constitute a transcription terminator. As expected, the 778-residue *E. faecalis* PBP1 (85.4 kDa) possesses all the characteristics of the multimodular bifunctional class A PBPs, i.e., five conserved motifs (motifs 1 to 5) in the glycosyltransferase module, 3 motifs (motifs 7 to 9) in the penicillin-binding module, and the conserved motif 6, designated the junction peptide (16, 17). A hydrophobic peptide, extending from residues 36 to 63, could serve as the primary anchor of PBP1 in the cytoplasmic membrane, which does not exclude the possibility that other internal peptides could also contribute to PBP1 interactions with the membrane.

The genome of *E. faecalis* V583 contains three class A PBP-encoding genes. Very recently, they were designated *ponA*, *pbpF*, and *pbpZ* (4). They encode PBPs similar to PBP1a, PBP2a, and PBP1b of *S. pneumoniae*. Three genes (*pbp5*, *pbpB*, and *pbpA*) code for class B PBPs: the low-affinity PBP5 involved in β -lactam resistance (12, 38) and two PBPs similar to PBP2x and PBP2b of *S. pneumoniae* (4).

Upstream of the *E. faecalis ponA* gene, one finds another ORF (nt 1052 to 1666) highly similar to *prfA* (or *recU* in *B. subtilis*), a gene that is regularly located upstream of *ponA* in different gram-positive bacteria as well as in *Mycoplasma* spp. (14, 34). That ORF codes for a 205-amino-acid protein (23.8 kDa) with a basic nature (theoretical pI value of 9.21) that confirms its similarity with the *B. subtilis* and *Bacillus stearothermophilus* PrfA, implicated in DNA repair and DNA recombination (14) and recently related to the restriction enzyme PvuII (37). No other ORF was detected on the same strand.

Overproduction and purification of a soluble *E. faecalis* PBP1 derivative. Attempts were made to overproduce a PBP1 soluble derivative (*PBP1) of *E. faecalis* JH2-2. The *ponA* gene, from which the initial 189 bp encoding the M1-A63 N-terminal peptide was deleted, was amplified by PCR from the genomic DNA and inserted into the pET28a(+) (Novagen,

VWR International, Leuven, Belgium) expression vector, yielding pDML1652, to produce a PBP1 bearing a hexahistidine sequence in its N-terminal extension (His*PBP1). Restriction and complete sequencing of the insert verified the construction. The His tag borne by the recombinant protein was clearly detected by Western blotting with the help of a mouse monoclonal anti-His₆ antibody conjugated with peroxidase (Roche-Diagnostics, Vilvoorde, Belgium; data not shown). However, it appeared as an insoluble product in *E. coli* HMS174DE3 cells (Novagen) transformed with pDML1652, grown at 37°C in Luria-Bertani medium containing kanamycin (50 µg/ml), and induced (at an optical density at 600 nm of 1.1) by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. To favor the production of a soluble recombinant protein, the culture was transferred at 18 to 20°C and then induced at that temperature for 22 h. Cells from a 1-liter culture were collected by centrifugation, suspended in a 50-ml mixture consisting of 50 mM KH₂PO₄-K₂HPO₄ (pH 8)-300 mM NaCl (buffer A) and 100 µM phenylmethylsulfonyl fluoride (Roche Diagnostics), and disrupted with a French press (SLM-Aminco, Rochester, N.Y.). Half of the preparation was loaded on a Zn²⁺-pentadentate chelator-agarose (Affiland, Liège, Belgium) column (25 ml) equilibrated against buffer A. The column was washed with the same buffer containing 5 mM imidazole until the A₂₃₀ was negligible. The His*PBP1 was eluted by a curvilinear imidazole gradient made by adding a 100 mM imidazole solution in buffer A (1 liter) to a constant volume (250 ml) of 5 mM imidazole in buffer A. Fifteen milligrams of 95% pure (estimated by densitometry; data not shown) His*PBP1 was obtained from a 1-liter culture. After the binding of fluorescent ampicillin (6'-Flu-amp) (25), the recombinant protein could be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, without any further treatment of the gel, with the help of a Molecular Imager FX laser scanner (Bio-Rad, Nazareth-Eke, Belgium). After dialysis against buffer A, the enzyme was stored at 4°C in the presence of 0.02% sodium azide.

Kinetic characterization of *E. faecalis* His*PBP1. (i) **DD-Peptidase/esterase activity.** Interactions with thiolester substrates such as benzoyl-Gly-thioglycolate and benzoyl-D-Ala-thioglycolate were studied as described previously (1, 18, 30). Hydrolysis of thiolester substrates ($\Delta\epsilon = -2,000 \text{ M}^{-1} \text{ cm}^{-1}$) by His*PBP1 (0.1 µM) was monitored over a 10-min period by measuring variations in absorbance at 250 nm on an UVIKON 860 apparatus (BRS, Ruisbroek, Belgium) coupled to a micro-computer via an RJ322 interface (23). Substrate concentrations varied from 100 µM to 5 mM. In some cases, the hydrolysis was monitored at 412 nm in the presence of 1 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] at a $\Delta\epsilon$ value of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$. The k_{cat}/K_m values were calculated from the initial rates of hydrolysis at low substrate concentrations and compared to those obtained with different solubilized HMM or LMM PBPs (PBP*s) (Table 2). The different PBP*s tested up to now display a large disparity of hydrolysis efficiencies on these two thiolester substrates. The LMM PBPs secreted by *Streptomyces* sp. strain R61 and *Actinomadura* sp. strain R39 are much more efficient than any of the HMM PBPs shown in Table 2. The hydrolysis of thiolester substrates performed in the presence of potential peptidic acceptors such as 5 or 10 mM D-Ala or Gly-L-Ala (Sigma-Aldrich, Bornem, Belgium)

TABLE 2. Hydrolysis parameters of thiolester substrates for different PBPs

PBP ^a	k_{cat}/K_m (M ⁻¹ s ⁻¹) for:		Reference or source
	Bz-D-Ala-Thg	Bz-Gly-Thg	
Class A			
His*PBP1 (<i>E. faecalis</i>)	230 ± 15	430 ± 20	This work
PBP1a* (<i>S. pneumoniae</i> R6)	128	5.7	8
PBP2a* (<i>S. pneumoniae</i> R6)	220	ND ^b	9
PBP1* (<i>M. leprae</i>)	4,500	ND	26
Class B			
PBP2x* (<i>S. pneumoniae</i> R6)	3,600	160	43
t-PBP3s* (<i>E. hirae</i>)	3,200	<20	1
PBP2b* (<i>S. pneumoniae</i>)	300	<20	1
PBP3* (<i>E. coli</i>)	100	20	2
LMM			
PBP R61 (<i>Streptomyces</i> sp. strain R61)	100,000	430,000	7
PBP R39 (<i>Actinomadura</i> sp. strain R39)	10,000	336,000	7

^a *, soluble derivative of the wild-type protein; t-PBP3s, tryptic derivative of PBP3s.

^b ND, not determined.

yielded slight but significant (1.6- to 2-fold) increases in k_{cat}/K_m , indicating that these acceptors were recognized and could be used in a transpeptidation reaction. The His*PBP1 did not show any hydrolytic activity either on monoacetyl- or diacetyl-L-Lys-D-Ala-D-Ala peptides or on benzoyl-Gly-phenyllactate (1, 18, 30).

(ii) **Affinity of the *E. faecalis* His*PBP1 for β-lactams.** The k_2/K parameter, accounting for the acylation step efficiency, was measured by monitoring the decrease of the intrinsic fluorescence of the His*PBP1 (15, 29). The experiments were performed at 37°C in 100 µl of 50 mM potassium phosphate buffer (pH 8)-300 mM NaCl, with an SLM-Aminco MC200 spectrofluorimeter. The excitation and emission wavelengths were 280 and 348 nm, respectively. The reactions were started by adding 3 µM His*PBP1, and the time-dependent decrease in fluorescence was recorded during 30 min. The values of the k_2/K parameter were obtained by plotting the apparent pseudo-first-order rate constant versus the antibiotic concentration. The k_3 parameter, accounting for the deacylation step, was estimated as follows. The His*PBP1 (10 µM) was incubated at 37°C for 45 min with 1 mM ampicillin, benzylpenicillin, or cephalothin. The excess of antibiotic was eliminated by successive steps of dilution and concentration, with a 0.5-ml Vivaspin concentrator (Vivascience, Sartorius AG, Goettingen, Germany). Brought back to the initial protein concentration, the samples were covered with mineral oil and incubated at 37°C for at least 20 h. Rates of breakdown of the 6'-Flu-amp/His*PBP1 complex and of binding of 6'-Flu-amp by the benzylpenicillin- or cephalothin-pretreated His*PBP1 were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and densitometry.

The binding of several β-lactam antibiotics, including ceftriaxone, whose high antibacterial activity against streptococci was noted 10 years ago (42), causes a quenching of the intrinsic fluorescence of the enzyme. The k_2/K and k_3 values obtained are presented in Table 3.

Most k_2/K values are characteristic of a low-affinity PBP

TABLE 3. Kinetic parameters for the interactions between His*PBP1 and various antibiotics

Antibiotic	k_2/K ($M^{-1} s^{-1}$)	k_3 ($10^5 s^{-1}$)
Benzylpenicillin	5.3 ± 1.6	<0.5
Ampicillin	11.3 ± 0.3	<0.5
Oxacillin	7.0 ± 2.6	ND ^a
Cephalothin	150.0 ± 1.8	<0.5
Cephalexin	3.2 ± 0.6	ND
Cephalexidine	10.0 ± 2.0	ND
Cefotaxime	43.0 ± 5.0	ND
Ceftriaxone	75.0 ± 10	ND

^a ND, not determined.

similar to those responsible for the enterococcal intrinsic β -lactam resistance. Indeed, the k_2/K values measured for the *Enterococcus hirae* PBP5, whose overproduction leads to an increased resistance to β -lactams, varied from 5 to 9 $M^{-1} s^{-1}$ (13), while that of the *E. hirae* PBP3r (a low-affinity PBP encoded by a large plasmid present in *E. hirae* S185) was 20 $M^{-1} s^{-1}$ (33, 36). The *E. faecalis* His*PBP1 is, however, slightly more sensitive to cephalosporins, in particular cephalothin and ceftriaxone, than to penicillins.

The MIC of benzylpenicillin for *E. faecalis* JH2-2 is 9 μM (3.2 mg/ml) (22). At such concentrations, the growth inhibition of JH2-2 cells could not be due to the inactivation of PBP1, as 240 min are needed to reach a half-saturation of this protein and as a generation time of 38 ± 1 min is measured for this strain in our culture conditions.

(iii) **Glycosyltransferase activity of the *E. faecalis* His*PBP1 (glycan chain synthesis).** Typical glycan polymerization assays were performed under the following conditions. *meso*-[¹⁴C]diaminopimelic acid (1.5 μM)-labeled lipid II (0.126 $\mu Ci/mol$) and 3.33 μM His*PBP1 were incubated at 30°C in a mixture containing 50 mM Tris-HCl, pH 7.5, 0.046% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 33 mM NaCl, 10 mM MgCl₂, 0.5% decyl polyethylene glycol, 12.5% 1-octanol, and 25% dimethyl sulfoxide. After 15-, 30-, or 60-min incubation periods, 30- μl samples were subjected overnight to chromatography on Whatman no. 1 filter paper in isobutyric acid–1 M ammonia (5:3) solvent (19). The radioactive compounds were detected by a Kodak phosphorus storage K screen and the Molecular Imager FX apparatus (Bio-Rad) using Bio-Rad Quantity One software. The polymerized radioactive peptidoglycan that remained at the origin on the chromatogram and the labeled lipid II migrating with the solvent were quantified by densitometry.

Incorporation of labeled precursors into polymerized peptidoglycan increased linearly versus time, reaching 1.4%. No polymerization was seen in the absence of enzyme or if the His*PBP1 was first incubated for 30 min at 37°C with 5 mM moenomycin. Previous transglycosylation assays performed with the same substrate and crude membrane preparations from *E. hirae* ATCC 9790 yielded 3.74% polymerized peptidoglycan.

These results indicate that the *E. coli* lipid II can be used as a substrate for transglycosylation by enterococcal enzymes, despite the differences existing in the structures of the pentapeptide units of the natural substrates.

Nucleotide sequence accession number. The EMBL accession number for the sequence of the 4.5-kb fragment bearing the *ponA* gene encoding *E. faecalis* JH2-2 reported in this paper is AJ302065.

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